

# Research Communication

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## *In-vitro* Study of *Moringa Oleifera* and *Casia Fistula* on Multi Drug Resistant Bacteria, Isolated from Bovine Mastitis Milk

**Abstract :** 'Mastitis' is the inflammation of udder cause mainly by bacterial infection. Antibiotics are used to treat bovine mastitis. For detection of mastitis milk, white side test (WST), california mastitis test (CMT) and pH were done. Multidrug resistant (MDR) bacteria were isolated by antibiogram-Assay. Leaf extracts of *Moringa oleifera* and *Cassia fistula* applied on the MDR samples. Mastitis milk showed positive results in WST, CMT, pH tests. These plant extracts reduced the bacterial count in  $10^3$  order.

Mastitis is a disease caused by multiple microorganisms. It has become one of the major problems of the dairy industries in almost all countries in the world. The physical, chemical and microbiological conditions of the milk changes due to mastitis<sup>1</sup>. Flakes (both fine and large) formation occurs in both acute and subacute mastitis. The pH of normal mastitis free milk is 6.4 to 6.6. In mastitis it may become alkaline upto 7.4 as sodium bicarbonate is formed<sup>2</sup>.

Mastitis can be classified according to the clinical signs. These include clinical, sub clinical, per acute and chronic mastitis. The major causative agents so far identified are bacteria like *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Mycoplasma sp*, *Corynebacterium sp*, *Escherichia coli*, *Klebsiella sp*, *Citrobacter sp*, *Serratia sp*, *Enterobacter sp*, *pseudomonas sp*, *Bacillus sp*, *Nocardia sp*<sup>3,4</sup>.

Somatic cells count is the indicators of the milk where the cows are suffering from clinical or subclinical mastitis due to the infection. Somatic cells of the milk consist of 75% leukocytes neutrophils, lymphocytes, macrophages, erythrocytes and 25% of epithelial cells only.<sup>5,6</sup>

Due to increase in population growth and economy in many countries from the middle of 1990s, consumption of milk in the world has been growing almost 10 million to 15 million tons per year. The milk production rate has also increased from 460 to almost 550 million tons in 2009.<sup>7</sup> The consumption of milk is highest in India, North America and Europe. Since 1980, the need of milk products is increasing in China, India and some other European countries<sup>7</sup>. The prevalence of clinical mastitis globally is 25% to 50% and in India an average of 50%. The prevalence of subclinical mastitis globally is 19% to 78% and in India an average of 17.5%.

Mainly four groups of antibiotics are used to treat bovine mastitis. These include the  $\beta$  lactams (e.g. penicillin and cephalosporin), tetracycline (e.g. oxytetracycline, tetracycline and chlortetracycline), aminoglycosides (e.g. streptomycin, neomycin and gentamycin) and Quinolones (e.g. Enrofloxacin or Ciprofloxacin)<sup>8</sup>. The drugs can be administered to animals by different routes like injection oral topical and by intramammary infusions with below the maximum residual limits (already mentioned above). Use of overdose antibiotic in animal food may have adverse effect on consumers health. Milk consumed with concentration above MRL may cause cancer<sup>8</sup> allergies. other injurious effects to human health<sup>9</sup>.

But dairy foods also have the properties to protect from cancer. Apart from using antibiotics, other methods may be used to cure mastitis. The alternative methods are : use of probiotics, means living microorganisms like lactic acid bacteria. The organisms are *Lactobacillus sp*, *Streptococcus sp*, *Bifidibacterium sp*, *Lactococcus sp*<sup>7</sup>. Use of Homeopathy Medicines such as the combination of *Phytolacca*, *Arnica*, *Conium* and *Ipecacuanha* are also reported<sup>10</sup>. Use of plant extracts like *Moringa oleifera* is also reported to reduce the inflammation. All leaf, pods and seeds of *Moringa oleifera* have anti inflammatory

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effect. These parts are also enriched in amino acids, different minerals and essential antioxidants.<sup>11</sup>

The crude ethanolic extract of the root was tested for its anti-inflammatory activity. Hydroethanolic extracts of *Moringa oleifera* inhibit the Nitric Oxide (NO) production in Lipopolysaccharides (LPS) induced inflammatory model. The crude extracts successfully induce the production of pro inflammatory mediators in LPS stimulated macrophages. The extract suppressed the expression of inflammatory mediators in LPS, and stimulated macrophages<sup>12</sup>.

*Casia fistula* is used as a herbal medicine because it has a great medicinal properties, like -anti-inflammatory, antifungal, antibacterial, laxative, hepatoprotective and wound healing property etc<sup>13</sup>.

The objectives of this present research are to study *in vitro* antibiogram on the bacterial isolates, which were collected from mastitis milk and to explore the *in vitro* antibacterial potential of sigre (*Moringa oleifera*) and Aragvadhā (*Cassia fistula*), which helps to reduce bacterial count.

**Materials and Methods : Sample collection and storage:** It is a cross sectional case control study. 30(thirty) milk samples were collected from Chakdah, West Bengal maintaining particular criteria. These milk samples were exposed to antimicrobial therapy and elapsed the withholding period of 30 days. Milk samples (15 ml) from each subject was collected in two sterile screw cap vials (UV irradiated) and transported to the laboratory in ice packed conditions.<sup>1</sup>

**Field screening:** The screening tests were done on fields for the identification of mastitis. Those tests were pH determination, White side Test (WST) and California Mastitis Test (CMT) and which were performed following the standard protocol<sup>2</sup>.

**Bacterial count and stock preparation:** The bacteriological count and stock preparation for further analysis were done by following the standard protocol<sup>14</sup>.

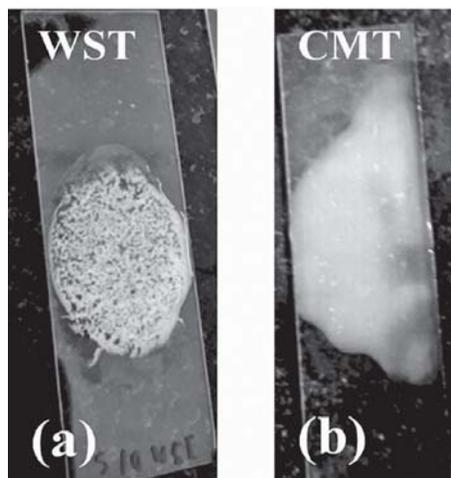
**Antibiogram by disc diffusion method as per CLSI rule:** Antibiogram of all isolated samples were done by following the recommendation of Clinical and Laboratory Standards Institute, 2014(CLSI,2014)<sup>15</sup>.

**Preparation of the plant aqueous extract through decoction method:** The leaf parts of *Moringa oleifera* (MO) and *Casia fistula* (CF) were used. Crude drug and water were taken in the ratio 1: 16 and then boiled in medium temperature and reduced upto 1/4 of total solution. Finally filtration process was done to make the decoction

of the crude drug. This test was done by following the conventional method.<sup>16</sup>

**Total viable count(TVC) or Log CFU/ml determination:** The bacterial culture had to be grown freshly in a shaker incubator until O.D<sub>600</sub> was 0.2. Then the bacterial culture was plated on the nutrient agar plates after the dilution was made upto 10<sup>-9</sup>. The bacterial colonies were found to be 5 × 10<sup>10</sup>. The next day again fresh culture was prepared from the control tube having O.D<sub>600</sub> 0.2. inoculum (0.5 μl) from the control tube was transferred to each of the tubes of each colony. 0.5 μl of culture contain 2 × 10<sup>7</sup> cells/ μl of bacterial cell. Another tube containing Luria broth was taken for control. Here tubes were chosen on the basis of turbidity. Spreading was done with spreader on the plate and incubated at 37 °C for overnight. Bacterial count of the control and leaf extracts of MO and CF applied tube were done. Compare the bacterial count and observe the order of decrease of bacteria. This test was done by following the method described by Yang et al, 2019<sup>17</sup>.

**Results : Mastitis screening test:** Results of the white side test are given in the figure 1a. The results of CMT tests of all samples are given in figure 1b. If sticky fluid is present, then the result is +, if the fluid is slimy then the result is ++, if the fluid becomes gelateneous then the result is +++. The pH of maximum of the samples were about 7.5. So mastitis was present.

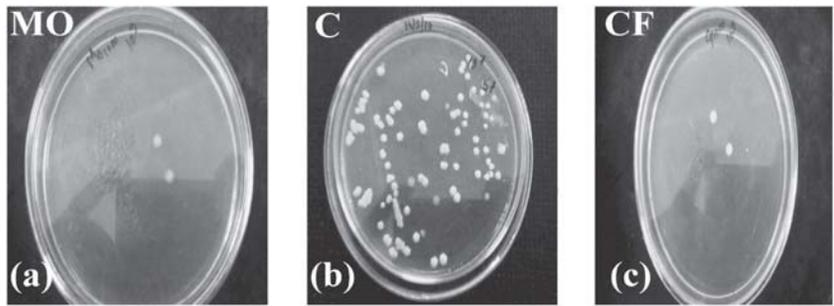


**Figure1:** (a) WST slide of mastitis (b) CMT slide of mastitis.

**Result of Microbiological Analysis : Result of bacterial count and antibiogram by disc diffusion method as per CLSI rules:** The results of bacterial count of all the 30 samples according to their respective dilutions (10<sup>-6</sup>, 10<sup>-7</sup>, 10<sup>-8</sup>) are given.

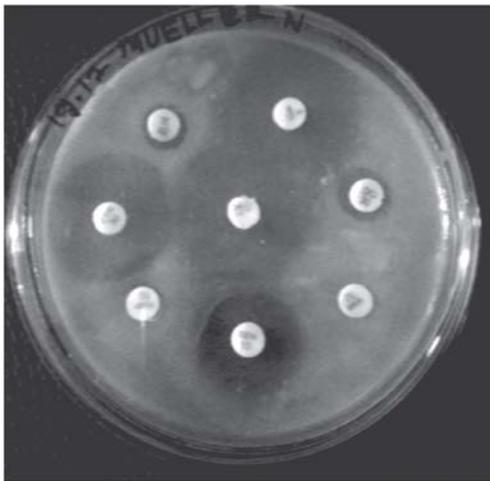
Isolated colony were taken for the antibiogram test to determine drug resistance property according to the

interpretation of CLSI, 2014. The results are given figure 2 and figure 3. The numbers of total samples were 30. In case of ampicillin, 14% of the tested organisms showed sensitivity against Ampicillin and 86 % of the tested organisms were resistant to the same drug. In case of Doxycycline 60% of the bacteria of the samples showed sensitivity to this drug, 32 % were resistant and 8% of the bacteria were intermediate. Bacteria (72%) of the sample showed sensitivity against Enrofloxacin, 20% bacteria were found to be resistant and 8% were intermediate organisms. Organisms 42% showed sensitivity to Ceftriaxone and the organisms 58% were resistant. In case of Gentamycin 68% of the tested organisms were sensitive to the drug, 16% were resistant and 16% were intermediate. 60% of the

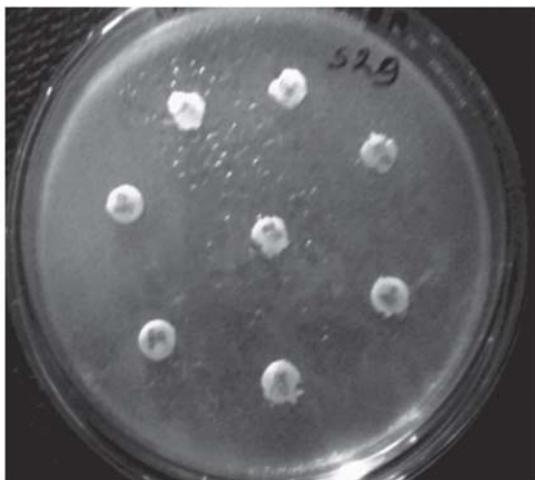


**Figure 4:** (b)Concentration of MDR bacterial cell in mastitis milk (a) Concentration of MDR bacterial cell in mastitis milk after spreading by plant extract(*Moringa oleifera*) and (c) Concentration of MDR bacterial cell in mastitis milk after spreading by plant *Casia fistula*

samples have tetracycline sensitive organisms, 32% were resistant and 8% were intermediate. All of the samples contained bacteria that were resistant to Amoxycillin. In case of Ciprofloxacin, 60% were sensitive, 20% were intermediate organisms.

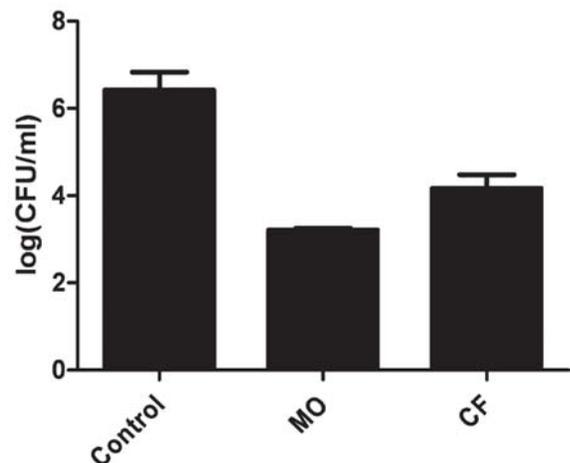


**Figure 2:** Mastitis milk sample containing bacteria showing zone of inhibition has formed around the antibiotic coated disc.



**Figure 3:** Mastitis milk sample containing bacteria showing resistance against drugs, zone of inhibition did not form around the antibiotic coated disk.

*Result of TVC or Log CFU/ml:* TVC test depicted that mastitis milk contained Multi Drug Resistant (MDR) bacteria in the range of  $1 \times 10^7$  to  $3 \times 10^7$  CFU/ml in figure 4b, which has been decreased to  $1.5 \times 10^4$  to  $2 \times 10^3$  after MO and CF leaf extract treatment to the same MDR organisms given in Figure 4a and Figure 4c. The result of the bacterial count after treating with the leaf extracts is given in the figure 5. It reduces the bacterial count to the order of  $10^3$  to  $10^4$ .



**Figure 5:** Total viable count in mastitis milk (Control), mastitis milk treated with *Moringa oleifera* (MO) and treated with *Casia fistula* (CF) extract. Milk samples were spread onto NA plates, bacterial colonies were counted after the plates were incubated at 37°C for 48hr. Datas are expressed as mean  $\pm$ SE.

**Discussions :** Mastitis is the inflammation of the udder. It has become one of the major problems of the dairy sector in almost all countries in the world. The physical, chemical and microbiological conditions of the milk changes <sup>11</sup>.Mastitis was confirmed by Positive CMT

and WST tests. Antibiogram results showed that all of the milk samples are having some MDR isolates with cumulative resistance to the four types of drugs used (Tetracycline, amoxicillin, ceftriaxone and enrofloxacin). Resistance against drugs mainly occurs due to the previous exposure of all the four types of drugs in field condition.

The use of these antibiotics in the field. These types of drugs are mainly used in the fields in the treatment of mastitis without assaying *in vitro* antimicrobial activity prior to treatment. Infusion of antibiotic ointments are usually used to treat mastitis of lactating cows through intra-mammary route. These treatments are expensive and problematic because of wide distribution of the multidrug resistant causative agents. Therefore, in this study, efficacy of herbal extracts were used as an alternative treatment. Different types of herbs are used for detoxifying, anti-inflammatory, and antibacterial activities<sup>15</sup>. Our MO and CF leaf extract prepared from two plants *Moringa oleifera* and *Casia fistula* which have been shown to have antibacterial activities, was the therapeutic agent of choice<sup>11,12</sup>. Our TVC test depicted that MDR bacterial counts are  $1 \times 10^7$  to  $3 \times 10^7$  CFU/ml for mastitis milk, where as bacterial count after MO and CF leaf extract treatment to the same MDR bacteria was  $1.5 \times 10^4$  to  $2 \times 10^3$  CFU/mL (Figure 5) respectively. So these extracts could be a good choice for mastitis treatment cow.

**Conclusion :** Antibiotics should be applied judiciously following the antibiogram even in the field condition. Milk should be supplied to the food chain after checking the drug residue and somatic cell count maintaining withhold period of mastitis treatment. Injudicious use of the antibiotics yields the problem of regarding the colonization of multidrug resistant bacteria which is also a public health concern. *In vitro* trial of unorthodox medicinal plant extract *Moringa oleifera* and *Casia fistula* paved the way toward future of evolving new therapeutics which may replace the trend of using antibiotics in case of mastitis and also opened the scope of alternative therapeutic research which may reduce the hazards of antibiotics. This pilot study warrants a long term prospective study to strengthen this view.

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